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Advances in Brief

Specific Tyrosinases Associated with Melanoma Replicative Senescence and Melanogenesis

Mary Strasberg Rieber and Manuel Rieber[1]

IVIC, Tumor Cell Biology Laboratory, Apartado 21827, Caracas 1020 A, Venezuela

Abstract

Replicative senescence occurs in normal cells, in contrast to their malignant counterparts which are generally immortal *in vitro*. We now show that induction of melanogenesis in subconfluent B16 melanoma cells deprived of growth factors can lead to irreversible growth arrest but continued cell viability, concurrent with the expression of specific glycosylated high molecular weight tyrosinases. These tyrosinase activities identify withdrawal from the cell cycle since they were not detected in reversibly arrested quiescent melanocytes, serum-deprived melanoma, or apoptotic melanoma. Our data suggest that different tyrosinases can distinguish cycling and noncycling cells of melanocytic origin and also imply that replicative senescence can be restored in some tumor cells when induced to terminal differentiation in the absence of growth-promoting agents.

Introduction

Irreversible growth arrest can occur by cellular senescence in normal cells, whereby they cease proliferation after a finite number of cell divisions (1). In contrast, malignant cells are immortal *in vitro*, and normal cells exposed to carcinogenic agents also show a prolonged life span in culture (2). Escape from senescence may be a genetically programmed event, partly due to mutations affecting expression of suppressor genes. Support for this theory stems from findings that normal cells can provide specific chromosomal fragments to suppress growth and favor senescence in tumor cells (3). However, irreversible growth arrest may require not only expression of suppressor genes but also modulation of their effects by external agents. Introduction of the p53 suppressor gene leads to myeloid leukemic cell death. However, this effect is inhibited by interleukin 6, which induces differentiation in these cells (4). Also, the senescence-inducing ability of X-chromosomes from late passages of normal hamster cells is increased by culture with the DNA-demethylating agent, 5-azacytidine (5). The latter findings suggest that growth control in normal and malignant cells is influenced not only by genetic information but also by extracellular factors and by the life span and stage or mode of differentiation, which may influence how cells respond to the environment.

Since cell-specific differentiation could become an alternative cancer therapy (6) and tyrosinase-associated melanogenesis is a predominant function of melanocytic cells and has potential as an antimelanoma target (7), we have now investigated conditions to optimize pigmentation and growth arrest in melanoma cells. We now show that melanogenesis, which derives from multiple conversions of L-tyrosine (7), can be induced in B16 melanoma exposed to high tyrosine levels (7). Moreover, we also show that serum starvation of melanoma cells prior to and during tyrosine treatment leads to irreversible growth arrest and expression of novel tyrosinase forms not detected in reversibly arrested melanocytes, in serum-starved melanoma, or in dying melanoma cells grown to high density without medium change.

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[1] To whom requests for reprints should be addressed. FAX: 582-501-1382.

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; L-DOPA, 3,4-dihydroxy-L-phenylalanine.

Materials and Methods

Cells and Tissue Cultures. These experiments were carried out with normal mouse Melan-A melanocytes, dependent on phorbol esters and serum to grow (8) and with a derivative of B16 melanoma BL6, both originally obtained from Dr. I. R. Hart (ICRF, London, United Kingdom). The latter was further selected in our laboratory for optimal induction of melanogenesis with 1–2 mM L-tyrosine for 24 h. Tyrosinase activity was assayed in cell extracts prepared in buffer S consisting of 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 mM EDTA, 60 mM β-glycerophosphate, 50 mM NaF, 0.1 mM sodium *o*-vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin. Lysates were frozen in dry ice-ethanol, thawed 3 times, and spun for 5 min at 14,000 rpm at 4°C. Whenever indicated, residues from lysates were resuspended in 30 μl of buffer P consisting of 2% SDS,[2] 0.1 M Tris-HCl (pH 6.8), and 1 mM phenylmethylsulfonyl fluoride and subjected to freezing and thawing 3 times. All other samples also received buffer P prior to electrophoretic separation by 10% SDS-polyacrylamide gel electrophoresis using highly purified SDS. After electrophoresis, gels were equilibrated in 0.1 M NaH_2PO_4, pH 6.8, at 37°C to remove SDS. Subsequently, assays involved transfer to the same buffer supplemented with 5 mM L-DOPA for incubation in the dark (9).

Whenever indicated, proteins solubilized in buffer S were reacted with wheat germ lectin-Sepharose (Catalogue no. L-6257; Sigma) for assay of glycosylation, by subsequent elution in 0.3 M *N*-acetylglucosamine in buffer S, for subsequent electrophoretic and enzymatic analysis.

Results

Effect of Serum and L-Tyrosine on B16 Melanoma Growth and Melanogenesis. In normal Dulbecco's medium supplemented with 10% fetal calf serum, subconfluent B16 melanoma cells grew exponentially until reaching confluence. Subsequently, monolayers were not maintained for more than 2 to 3 days, since they started to detach, became lightly pigmented, and died, as shown by others (10). Cells detaching at high confluence revealed DNA fragmentation like that found in apoptotic cells (not shown).

Serum-supplemented cells treated with 2 mM tyrosine showed increase in pigmentation concurrent with growth arrest but only partial cell detachment and death. However, maximal melanogenesis and survival was detected in subconfluent cultures deprived of serum for 28 h and then exposed to L-tyrosine for 28 h in serum-free medium. Table 1 shows that serum depletion in the presence of L-tyrosine promotes maximal melanogenesis suggesting that growth factors in serum decrease basal pigmentation, as implied by others (10). This was further substantiated in Table 1 which showed much greater melanogenesis when promoted by L-tyrosine in the serum-starved cells. Moreover, the latter cells stopped dividing and remained as a highly pigmented monolayer for several weeks.

Assays of cell viability revealed trypan blue uptake and lack of retention of neutral red in highly confluent cultures which began to detach and die after growth in medium supplemented with 10% serum. This behavior was in marked contrast with growth-arrested pigmented cultures induced with L-tyrosine in serum-free medium which resembled unpigmented proliferating melanoma (not shown). The latter cells remained pigmented and firmly attached but failed to divide

Table 1 *Relative melanin content in B16 melanoma cells*

Melanoma content was determined in duplicates in cells detached with a rubber policeman in phosphate-buffered saline. Aliquots were removed for cells and protein quantitation, and cell pellets were extracted with 0.5 N NaOH at 60°C for 30 min for subsequent melanin analysis by comparison with a synthetic standard, similarly dissolved.

Cell culture	μg melanin/million cells
Medium plus 10% serum for 48 h	0.021 ± 0.03[a]
Medium with no serum for 48 h	0.131 ± 0.002
Medium plus 10% serum plus L-tyrosine for 28 h	0.340 ± 0.007
Cells deprived of serum for 28 h and treated with L-tyrosine for a further 28 h	2.928 ± 0.020

[a] Mean ± SD.

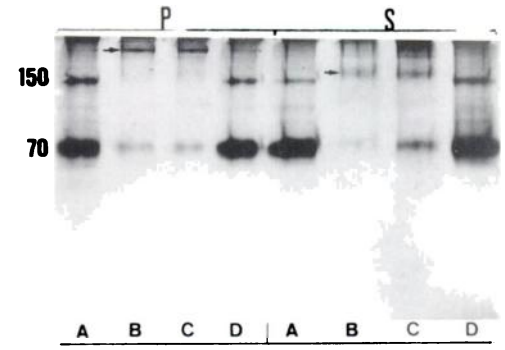

Fig. 1. Decrease in M_r 70,000 tyrosinase and expression of slow-migrating tyrosinases in irreversibly arrested cells. Melanoma extracts were prepared, electrophoretically separated, and assayed for tyrosinase from B16 melanoma as described under "Materials and Methods." *A*, extract from cells maintained without serum for 48 h; *B*, extract from cells maintained without serum for 72 h with addition of 2 mM tyrosine during the last 48 h; *C*, as in *A*, but with addition of 2 mM tyrosine during the last 24 h; *D*, extract from subconfluent proliferating cells kept in serum-supplemented medium with no additional tyrosine. *Lanes* under *P* are from particulate fractions and *lanes* under *S* originate from soluble fractions. *Ordinate*, approximate molecular weights of tyrosinases; *arrows*, activities preferentially detected in irreversibly arrested cells.

even when provided with serum-supplemented medium devoid of L-tyrosine, suggesting that they had reached replicative senescence and terminal differentiation.

Cells in which reversible growth arrest was induced by a 48-h serum deprivation showed a major M_r 70,000 tyrosinase activity like that seen in proliferating cells, but with a greater ratio of a M_r 55,000 tyrosinase to that of the major M_r 70,000 tyrosinase (Fig. 1). Hence, it appears that the M_r 70,000 tyrosinase activity is detected as a main component in unpigmented growing cells, and this tyrosinase is partly decreased in melanoma cultures starved of serum for 48 h. In contrast, we detected M_r 200,000 and 150,000 tyrosinases and an even greater decrease in the M_r 70,000 tyrosinase in irreversibly arrested highly pigmented cells. The M_r 200,000 tyrosinase appeared primarily in an insoluble form, in contrast to the M_r 150,000 tyrosinase which was detected preferentially in soluble form (Fig. 1). Additional experiments with quiescent cells were undertaken to define whether the M_r 200,000 and 150,000 tyrosinases were also associated with reversible growth arrest. This was undertaken using normal melanocytes which require both tumor-promoting agents and serum to proliferate (8) but enter into a reversible quiescence upon removal of growth-promoting agents (8). Normal melanocytes deprived for 6 days of serum and phorbol esters showed a major M_r70,000 tyrosinase (Fig. 2, *Lanes A* and *B*) which decreased in tyrosine-treated cells that revealed increased pigmentation in 30% compared to the same untreated cells (not shown).

Serum-stimulated melanocytes showed greater detection of the M_r 55,000 tyrosinase and a concurrent decrease in the M_r 70,000 isoform. A comparison of proliferating melanocytes and melanoma cells revealed a much higher ratio of the M_r 70,000 tyrosinase to that of the M_r 55,000 tyrosinase in growing melanoma cells, and a marked decrease of the M_r 70,000 activity with melanoma irreversible arrest and pigmentation (Fig. 2, *C–F*). The latter cells also showed slow-migrating tyrosinases not detected in quiescent melanocytes deprived for 6 days of serum and tumor promoters (Fig. 2, *A* and *F*).

Tyrosinases from Senescent Cells Are More Glycosylated Than Those from Apoptotic or Proliferating Cells. Tyrosinase is known to be synthesized as a M_r 55,000 molecule which is glycosylated to give heterogeneous mature M_r 70,000 enzyme (11). Hence, we investigated whether the tyrosinases from melanoma cells which underwent heavy pigmentation and reached replicative senescence differed in glycosylation from those in subconfluent proliferating cells and those in highly confluent cultures which started to detach and die after reaching confluence and becoming partly pigmented, as shown by others (10). For this analysis, soluble extracts were reacted with WGA-Sepharose (Sigma), followed by extensive washing in solubilization S buffer and elution in the same buffer containing 0.3 M *N*-acetylglucosamine, to elute bound glycoproteins. Subsequently, flow-through nonglycosylated proteins and eluted fractions were electrophoretically separated and assayed for tyrosinase activity. This showed that proliferating cells and those detaching at high confluence have quite similar tyrosinases and that both cell types show M_r 55,000 and 70,000 forms unable to bind to WGA-Sepharose, whereas no comparable tyrosinases were found in irreversibly arrested adherent cells. The latter showed only WGA-binding tyrosinases, including

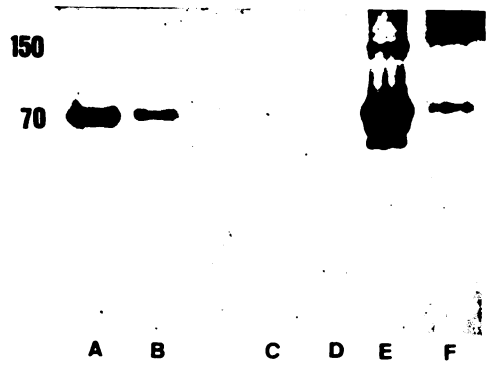

Fig. 2. Comparison of tyrosinase activities in melanocytes and melanoma cells. Extracts were prepared from normal melanocytes and melanoma cells for fractionation and assay as described in Fig. 1. *A*, *B*, soluble extracts from quiescent melanocytes kept without serum or phorbol esters for 6 days; *B*, as in *A* with addition of 2 mM tyrosine; *C* and *D*, extracts from 6-day quiescent melanocytes stimulated for 20 h with 10% serum and 160 nM tetradecanoylphorbol acetate, including 2 mM L-tyrosine in *D*; *E*, extracts from subconfluent proliferating melanoma cells in serum-supplemented medium with no additional tyrosine; *F*, extract from subconfluent melanoma cells maintained for 48 h without serum with addition of 2 mM L-tyrosine during the last 24 h.

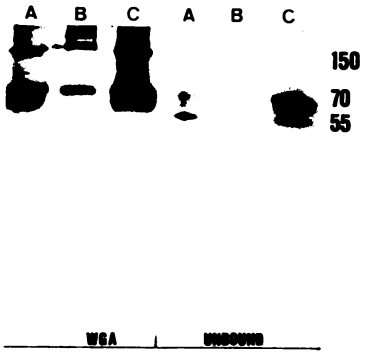

Fig. 3. Lack of nonglycosylated tyrosinases in irreversibly arrested melanoma cells. Glycosylated tyrosinases were separated from their nonglycosylated counterparts by specific elution from WGA-Sepharose as described under "Materials and Methods." *A*, soluble fraction from cells detaching at high confluences in serum-supplemented medium in which DNA fragmentation ladders are detected; *B*, soluble fraction from irreversibly arrested attached cells starved of serum for 48 h and incubated for the last 24 h with 2 mM tyrosine; *C*, soluble fraction from proliferating attached cells grown in serum-supplemented medium. *Left*, WGA-binding tyrosinases; *right*, unbound tyrosinases.

slow-migrating forms and decreased levels of the M_r 70,000 tyrosinase, which differed from those seen in WGA-binding fractions from the other cell types (Fig. 3) and resemble those shown in the soluble fraction from similar extracts not treated with WGA-Sepharose (Figs. 1 and 2).

Hence, it seems that subconfluent proliferating melanoma cells or those that reach high densities and start to detach and die concurrently with DNA fragmentation have similar tyrosinases. These differ from those found in melanoma cells that enter replicative senescence and can remain tenaciously attached and heavily pigmented for more than 7 weeks.

Discussion

Induction of melanin synthesis in melanoma cells generally correlates with inhibition of cell growth (12). L-Tyrosinase is a key enzyme in melanin synthesis in mammals (7) and this pigmentation generates a number of reactive intermediates which are know to promote melanocytotoxicity (7, 13).

We now show that induction of melanogenesis in the absence of growth factors present in serum does not lead to cytotoxic effects but rather to heavy pigmentation and withdrawal from the cell cycle in cultures which resemble terminally differentiated senescent cells. This was paralleled by a paradoxical decrease in the M_r 70,000 tyrosinase activity which is the major tyrosinase in unpigmented melanocytes and melanoma cells. This is compatible with reports of high tyrosinase activity in albino oocytes (14), as well as melanocyte-stimulating hormone induction of growth arrest and tyrosinase without melanogenesis (15). Moreover, irreversible growth arrest and pigmentation were paralleled by expression of novel tyrosinase isoforms with molecular weights of 200,000 and 150,000, not detected in reversible arrested cells. This suggests that the M_r 70,000 tyrosinase activity could be involved with early stages of tyrosine hydroxylation to give L-DOPA, and L-DOPA oxidase activity (14, 15), but it may not be the only enzyme in the final stages of melanogenesis that produce the particulate melanin polymer (16).

Melanogenesis associated with subconfluent irreversibly arrested cells was found to be dependent on high tyrosine levels but independent of serum, producing both secreted and cell-associated pigmentation (not shown). This differed from earlier reports indicating a serum requirement for the secretion of pigmentation in confluent melanoma cells (17).

Another characteristic of the highly pigmented senescent melanoma cells now described is their very strong attachment to substratum, compared to that of serum-supplemented proliferating cells and reversibly arrested serum-starved cultures. Both of the latter types rapidly detached within 5 to 10 min of trypsinization whereas the senescent cells resisted twice as much trypsin for 30 min without significant cell release from the substratum (not shown).

This stronger attachment may be due to the fact that senescent cells are known to expose unique epitopes on fibronectin, which may contribute to their firmer attachment (18).

It remains to be further established whether the novel tyrosinase now described in irreversibly arrested cells represent differentially processed forms (16) of the glycosylated enzyme present in uninduced cells or rather result from alternative splicing of a common primary transcript (19) or from more than one gene product with tyrosinase activity (11, 20). Nevertheless, we are currently purifying the tyrosinases from terminally differentiated pigmented cells to compare them with those detected in cycling melanocytes and melanoma. However, at this stage, it seems apparent that the novel tyrosinases now described could have some potential application in diagnosis and follow-up therapy if they can distinguish melanoma cells capable of reentering the cell cycle and those that have taken the terminal differentiation pathway.

These studies also show that induction of cell-specific differentiation in the absence of incompatible growth-promoting signals can lead to restoration of permanent growth control in some tumor cells.

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